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13. ABSTRACT (Maximum 200 Words) Chromosome 11q13 is amplified in 13% of primary breast cancers and is indicative of a poor prognosis (1). The region amplified includes the cyclin D1 gene, hst-1, int-2, and ems1 (cortactin); however, only cyclin D1, a cell cycle regulator, and cortactin, a cytoskeletal protein, are overexpressed. Several studies have shown increased levels of these proteins in different breast cancer cell lines and their amplification is associated with a more invasive phenotype; however whether or not these proteins are directly contributing to the enhanced tumorigenic potential has not been clearly addressed (2). While the function of cyclin D1 is fairly well understood very little is known about the function of cortactin overexpression in mammary tumorigenesis. The goals of this proposal are to 1) understand the function of cortactin in normal cell growth and differentiation; 2) determine the consequences of cortactin overexpression in mammary epithelial cells; and 3) identify cellular targets of cortactin. These goals will be achieved using genetic, biochemical, and cell biological approaches including generation of mice carrying a targeted disruption in the cortactin gene, derivation of mammary epithelial cells overexpressing wild type and mutant cortactin, and analysis of a two-hybrid screen using full length cortactin.				
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INTRODUCTION

Chromosome 11q13 is amplified in 13% of primary breast cancers and is indicative of a poor prognosis (1). The region amplified includes the cyclin D1 gene, hst-1, int-2, and ems1 (cortactin). Int-2 and hst-1 are rarely overexpressed in these tumors while both cyclin D1, a cell cycle regulator, and ems1 (also called cortactin), a cytoskeletal protein, are overexpressed. Several studies have shown increased levels of these proteins in different breast cancer cell lines and their amplification is associated with a more invasive phenotype; however whether or not these proteins are directly contributing to the enhanced tumorigenic potential has not been clearly addressed (2). While the function of cyclin D1 is fairly well understood very little is known about the function of cortactin overexpression in mammary tumorigenesis. The goals of this proposal are to 1) understand the function of cortactin in normal cell growth and differentiation; 2) determine the consequences of cortactin overexpression in mammary epithelial cells; and 3) identify cellular targets of cortactin. These goals will be achieved using genetic, biochemical, and cell biological approaches including generation of mice carrying a targeted disruption in the cortactin gene, derivation of mammary epithelial cells overexpressing wild type and mutant cortactin, and analysis of a two-hybrid screen using full length cortactin.

BODY

The original specific aims were as follows:

- I. To Determine the Function of Cortactin in Normal Cell Growth and Differentiation.**
- II. To Directly Determine the Role of Cortactin on Mammary Epithelial Cell Growth and Differentiation.**
- III. To Identify Proteins Interacting with Cortactin.**

Specific Aim I

- I. To Determine the Function of Cortactin in Normal Cell Growth and Differentiation.**

Previously, we had generated a targeting vector for the cortactin gene. Using this vector we showed that disruption of a single allele of cortactin in the ES cell line, AK7, resulted in an altered cell phenotype. ES cells which are heterozygous for the cortactin gene, differentiate into a population of cells of unknown origin. Not surprisingly, these cells are unable to contribute significantly to the germline. As reported last year, we have used a different ES cell line, to target the cortactin locus. J1 or TC1 ES cells which are heterozygous for cortactin, do not differentiate. We injected 4 TC1 clones and 2 J1 clones and have obtained chimeras. One of the J1 clones resulted in 80% chimerism while poor results were obtained with the TC1 clones. The 80% chimera is being bred to obtain germline transmission. We are also injecting additional J1 clones to generate multiple cortactin +/- line. This is important to assure that any phenotype observed is really due to loss of cortactin.

In addition to generating these mice, we are also trying to make ES cells which are homozygous null for cortactin. To this end, we have started to re-target some of the J1 cortactin +/- ES clones. The original vector is diagrammed below. The presence of the

---- SabgeobpA-----DTApgk

-----: homologous arm; SA: splice acceptor; β geo: β -gal-neo fusion; bpA: bovine poly A
DTApgk:diphtheria toxin A subunit driven by phosphoglucokinase promoter

phage LoxP sites, allows for site specific deletion. LoxP sites are recognized by the Cre recombinase and sequences flanked by these sites are excised in a cre-dependent recombination reaction. Therefore, treatment of the J1 cortactin +/- ES cells with Cre will

result in deletion of the SA β geo bPA sequences. Cells will be screened for the deletion by PCR and confirmed by Southern. We will then re-target these cells with our original targeting vector and screen for homozygous cortactin null cells by PCR with re-confirmation by Southern blotting. Using this strategy, we are currently screening for the homozygous null cells. These cells can be used not only for in vivo chimera experiments or in vitro differentiation studies, but also will be useful for biochemical and cell biological studies on understanding

Our second approach is to generate a conditional knockout of the cortactin gene. As it is anticipated, based on the ES cell results, that cortactin may be required during embryogenesis, the conditional knockout will be extremely useful for understanding cortactin function in mammary epithelial biology. In addition, our hope is to be able to look at how loss of cortactin may affect mammary tumors induced by Neu. Currently, we are in the process of making the vector for the conditional knockout. We anticipate that within the next year we will have these mice and will be in the process of analyzing them.

Specific Aim II

II. To Directly Determine the Role of Cortactin on Mammary Epithelial Cell Growth and Differentiation.

One of the major goals of this proposal was to determine whether overexpression of cortactin or variants of cortactin affected mammary epithelial biology. To this end we have used an immortalized cell line, Scp2, which can mimic some of the morphogenetic events that occur in vivo (3). When placed in an appropriate extracellular matrix in the presence of lactogenic hormones, these cells form a polarized three-dimensional luminal structure that is capable of secreting milk proteins into the lumen. Using these cells we have shown that expression of full length cortactin results in a number of phenotypic changes compared to control cells. These include a "scattered" cell morphology, an increase in cell migration, decreased milk production, and alterations in polarity. Cells expressing full length cortactin have an altered cell morphology. While control cells form tight epithelial colonies, cortactin expressing cells are more refractile. This is typical of cells that are more migratory. Consistent with this phenotype, these cells are able to migrate and close a wound faster than the control cells. The cells, however, do not appear to be more invasive. Since invasion is likely to be a late event, we may look at the consequences of overexpression in tumorigenic lines such as T47D cells.

While cortactin does not induce SCp2 cells to become invasive, it does affect the differentiation program of these cells. When the cells are plated in matrigel and given lactogenic hormones, they organize themselves into alveolar like structures and produce milk proteins such as b-casein. Expression of cortactin resulted in a dramatic decrease in b-casein positive cells. In addition, when we analyzed the organization of the alveolar structure, there was a loss of polarity as judged by a loss of E-cadherin and b-catenin

localization and a dramatic decrease in the junctional protein ZO-1. Limited effects are seen when we look at a human mammary cell line, MCF10A. This could be due to differences in species or alternatively the tissues from which they were derived. In any case, the *in vitro* results strongly suggest that amplification of the cortactin gene is likely to contribute to the progression of mammary tumors.

To determine what regions of cortactin may be important for this phenotype, a number of mutants were made. Originally we had hoped that expression of the N-terminal half or C-terminal half may in some way interfere with cortactin function. However, our results suggest that expression of the full length, N-terminal or C-terminal half of cortactin in SCP2 results in essentially the same phenotype. This raises the possibility that these two major halves of cortactin are able to either signal independently to induce these changes or alternatively, can activate the endogenous cortactin protein. Interestingly, both proteins are able to localize to the cortical cytoskeleton, the region of the cell in which cortactin is normally found. Generation of the knockout cells as well as the studies in specific aim III will help to better address which of these models can explain our results.

Given that the N-terminal and C-terminal halves of cortactin can alter the properties of SCP2 cells and MCF10a cells, we have made additional mutations to see if we can suppress these phenotypes. The N-terminal half of cortactin contains a repeat region which can bind F-actin (4). The fourth repeat has been shown to bind to phosphatidylinositol lipids which have been implicated in regulating cell migration (5). Cells expressing this mutant no longer affect the migratory or differentiation properties of the SCP2 cells. Similarly disruption of the Src Homology 3 (SH3) domain in the C-terminal half of cortactin also blocks the ability of the C-terminal half to alter SCP2 cells. Thus, these regions may somehow contribute to the deregulation of cell migration and/or differentiation in mammary epithelial cells and potentially be activating pathways normally regulated by cortactin. We are currently trying to wrap up these studies and prepare a manuscript. Based on this work, we plan to take these studies *in vivo* by making MMTV-cortactin transgenic mice. These mice should express cortactin in the mammary gland. We have made the construct and are currently preparing it for injection. These studies should provide an interesting *in vivo* model for looking at the consequences of cortactin overexpression. Studies with these animals will include crosses with different mammary tumor models to determine whether cortactin overexpression makes the tumors more metastatic.

Finally, we have also begun to look at potential cooperativity between cortactin and cyclin D1. As indicated above, cyclin D1 is the other gene in this locus which also has elevated protein levels. Our analysis in MCF10A cells indicates that overexpression of cortactin along with cyclin D1 appears to dramatically affect mammary differentiation resulting in disorganized structures. Future studies will look at mutants of cortactin along with cyclin D1.

Specific Aim III

III. To Identify Proteins Interacting with Cortactin.

The third major goal of this proposal was to identify proteins interacting with cortactin. We have used a number of approaches including yeast two hybrid screens and small cDNA pool screens. In addition, we have taken advantage of the identification of the optimal binding sequence of the SH3 domain and used a bioinformatics approach. This has yielded the identification of a multi-domain Rho family GTPase exchange factor, facio-genital dysplasia gene 1 (FGD1) as a binding protein for cortactin. Given that expression of cortactin enhances cell migration and that its localization is known to be regulated by a Rho family member, Rac, FGD1 is likely to be an important effector/regulator of cortactin

function (6). We have obtained the FGD1 cDNA and are currently testing its role in cortactin biology. A second protein that has been identified is the Wiskott-Aldrich syndrome interacting protein, Wip, another regulator of the actin cytoskeleton (7). In collaborative study with Raif Geha's laboratory at Children's hospital/Harvard Medical school, we have shown that wip binds to the cortactin SH3 domain. During the next year we will test the functional significance of cortactin's interaction with wip and FGD1 using the Scp2 cells or MCF10a mammary cell systems. These studies should provide important incites into the pathways by which excess cortactin expression may contribute to mammary tumorigenesis in vivo

KEY ACCOMPLISHMENTS

- Generation of ES cell lines which are heterozygous and potentially homozygous for the cortactin gene.
 - Generation of high percentage chimeras from these ES cells
 - Discovery that expression of cortactin and different variants of cortactin can alter the properties of mammary epithelial cells and that cortactin can cooperate with cyclin D1 to dramatically disrupt mammary differentiation.
 - Generation of a MMTV-Cortactin vector for transgenic studies.
- Identification of FGD1 and Wip as cortactin binding proteins.

REPORTABLE OUTCOMES

Manuscripts: None. We are in the process of writing up the work described in specific Aim II

Presentations: University of North Carolina, Cell and Developmental Biology Dept.
(no abstract available)

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CONCLUSIONS

We have made progress on all three specific aims. Over the next year we hope to make considerable progress towards understanding the role of cortactin in vivo by analyzing cortactin deficient mice and by generating a conditional knockout of cortactin. We will also generate a MMTV-cortactin transgenic. We will continue to pursue our in vitro studies in the mammary epithelial cell lines and by looking at additional cortactin mutants, known regulators and effectors, and looking at interacting proteins identified in specific aim III, we should gain a better understanding of how expression of cortactin contributes to mammary tumor progression. Finally, the data obtained thus far, should provide us with the necessary preliminary information needed to pursue additional funding for these studies.

REFERENCES

1. Schuuring, E., E. Verhoeven, H. van Tinteren, J. L. Peterse, B. Nunnink, F. B. Thunnissen, P. Devilee, C. J. Cornelisse, M. J. van de Vijver, W. J. Mooi, and et al. 1992. - Amplification of genes within the chromosome 11q13 region is indicative of poor prognosis in patients with operable breast cancer. *Cancer Res.* 52:5229-34.
2. Schuuring, E. 1995. - The involvement of the chromosome 11q13 region in human malignancies: cyclin D1 and EMS1 are two new candidate oncogenes--a review. *Gene.* 159:83-96.
3. Weaver, V. M., A. R. Howlett, B. Langton-Webster, O. W. Petersen, and M. J. Bissell. 1995. - The development of a functionally relevant cell culture model of progressive human breast cancer. *Semin Cancer Biol.* 6:175-84.
4. Wu, H., and J. T. Parsons. 1993. - Cortactin, an 80/85-kilodalton pp60src substrate, is a filamentous actin-binding protein enriched in the cell cortex. *J Cell Biol.* 120:1417-26.
5. He H, Watanabe T, Zhan X, Huang C, Schuuring E, Fukami K, Takenawa T, Kumar CC, Simpson RJ, Maruta H. Role of phosphatidylinositol 4,5-bisphosphate in Ras/Rac-induced disruption of the cortactin-actomyosin II complex and malignant transformation. *Mol Cell Biol.* 1998 Jul;18(7):3829-37
6. Weed SA, Du Y, Parsons JT. 1998. Translocation of cortactin to the cell periphery is mediated by the small GTPase Rac1. *J Cell Sci.* 111 (Pt 16):2433-43.
7. Ramesh N, Anton IM, Hartwig JH, Geha RS. 1997 WIP, a protein associated with wiskott-aldrich syndrome protein, induces actin polymerization and redistribution in lymphoid cells. *PNAS.* 94(26):14671-6.

APPENDICES.

NONE.